Felid herpesvirus-1 infection: influence on proinflammatory cytokine and toll like receptor expression in vitro

Abstract

Felid herpesvirus 1 (FeHV-1) causes an acute respiratory illness known as feline rhinotrachetitis. Moreover, it is associated with several syndromes in the feline eye including conjunctivitis, epithelial keratitis, herpes stromal keratitis (HSK) and corneal sequestration. The aim of this study was to gain first insights into the interaction between FeHV-1 and the feline innate immune system, specifically in peripheral blood mononuclear cells (PBMC). FeHV-1 infection in Crandell Reese Feline Kidney cells (CRFK) as well as in feline PBMC resulted in the induction of proinflammatory cytokine mRNA, i.e. for Interleukin-6 (IL-6), Interleukin-1 (IL-1) and Tumor necrosis factor alpha (TNF α). To evaluate a potential role of Toll like receptors (TLR) in the infection with FeHV-1, the level of TLR mRNA in non-infected as well as in infected feline cells was measured. Both TLR 2 and 3 mRNA were down-regulated in FeHV-1-infected cells. Results concerning the expression of TLR 9 mRNA were inconsistent, showing up-regulation in some instances and down-regulation in other instances. TLR 7 was clearly up-regulated upon infection of CRFK cells with FeHV-1, not only on the mRNA level but also in terms of function. Intriguingly, the same effect was not observed in feline PBMC. Thus, the interaction between FeHV-1 and the innate immune system proceeds in a celltype-specific manner.

Key words: Felid herpesvirus 1 / innate immunity / Toll like receptor.

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Arbeit unter der Leitung von Dr. A. Vögtlin

Felid herpesvirus-1 infection: Influence on Proinflammatory cytokine and Toll like receptor expression *in vitro*

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for the legless hens of my grandfather!

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1. Abstract

Felid herpesvirus 1 (FeHV-1) causes an acute respiratory illness known as feline rhinotrachetitis. Moreover, it is associated with several syndromes in the feline eye including conjunctivitis, epithelial keratitis, herpes stromal keratitis (HSK) and corneal sequestration. The aim of this study was to gain first insights into the interaction between FeHV-1 and the feline innate immune system, specifically in peripheral blood mononuclear cells (PBMC). FeHV-1 infection in Crandell Reese Feline Kidney cells (CRFK) as well as in feline PBMC resulted in the induction of proinflammatory cytokine mRNA, i.e. for Interleukin-6 (IL-6), Interleukin-1 (IL-1) and Tumor necrosis factor alpha (TNF α). To evaluate a potential role of Toll like receptors (TLR) in the infection with FeHV-1, the level of TLR mRNA in non-infected as well as in infected feline cells was measured. Both TLR 2 and 3 mRNA were down-regulated in FeHV-1-infected cells. Results concerning the expression of TLR 9 mRNA were inconsistent, showing up-regulation in some instances and down-regulation in other instances. TLR 7 was clearly up-regulated upon infection of CRFK cells with FeHV-1, not only on the mRNA level but also in terms of function. Intriguingly, the same effect was not observed in feline PBMC. Thus, the interaction between FeHV-1 and the innate immune system proceeds in a celltype-specific manner.

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2. Introduction

2.1. *Herpesviridae*

Herpesviruses are large, double stranded DNA viruses with an icosahedral capsid surrounded by a tegument layer and an envelope [34]. Today, three families build the order of *Herpesvirales*. The first family named *Herpesviridae* contains mammal, bird and reptile viruses and is further subdivided into three subfamilies, i.e. *Alpha-*, *Beta-*, and *Gammaherpesvirinae*. The second family called *Alloherpesviridae* includes fish and frog viruses, and a bivalve virus belongs to the third family *Malacoherpesviridae* [28].

Infections with members of the *Alphaherpesvirinae* are characterized by a short and productive replication cycle in cell culture leading to a typical cytopathic effect [34]. Important members of the *Alphaherpesvirinae* are the herpes simplex virus type 1 (HSV-1, causing herpes labialis) of humans, the Bovine herpesvirus 1 (BoHV-1, causing infectious rhinotracheitis in cattle), the Porcine herpesvirus (Aujeszky's disease), and the Equine herpesviruses 1 and 4 (EHV-1 and EHV-4, causing respiratory disease and abortion in horses) [31, 66, 68, 72]. Typical for *Betaherpesvirinae* is a slow productive replication cycle and their potential to generate giant cells [34]. The Cytomegalovirus (CMV) causing CMV-pneumonia is a typical member of the *Betaherpesvirinae*. The productive replication cycle time of the family of the *Gammaherpesvirinae* is very heterogeneous. For humans Epstein-Barr-Virus (EBV) involved in Morbus Hodgkin and Morbus Pfeiffer disease belongs to this group [60]. Among the gammaherpesviruses of Veterinary interest are the Alcelaphine herpesvirus 1 (causing bovine malignant catarrhal fever), the Equine herpesvirus 2 (EHV-2, causing respiratory disease and eye diseases like keratitis and conjunctivitis) and the ovine herpesvirus 2 (OvHV-2, causing malignant catarrhal fever) [2].

The replication of the herpesviruses takes place inside the nucleus of the infected cell [69]. Some antigens are shared within the family of *Alphaherpesvirinae*, but different animal

species have distinct envelope glycoproteins. In general, the natural host range is highly restricted and most herpesviruses have evolved in association with single host species, but occasional transfer to other species can occur in nature [8].

An important biological feature of herpesviruses is the establishment of lifelong persistence in a latent state following primary infection [63]. At the primary site of infection lytic replication of the virus takes place leading to the death of infected cells. Subsequently, close-by sensory neurons are invaded by the progeny viruses [78]. These viral particles move along the nerves towards sensory ganglia, in which they establish latency [64]. Typical for the latent viral genome is an extrachromosomal phase with only a restricted portion of genome transcription [69]. The latently infected cells stay intact and the virus DNA is protected from the immunological host response [82]. The consequences are that the infected animal is persistently infected and is not able to eliminate the virus. Different factors including stress and treatment with immunosuppressive agents can lead to a reactivation of the infection [82]. For the virus transmission a close contact between individuals is needed, as the virus is not stable in the environment [84]. In the mostly cases the first contact is often between mother and offspring. Because of the persistently infection and the earlier infection time point leads this to a wide prevalence of the virus [84].

2.2. Felid herpesvirus-1

Felid herpesvirus 1 (FeHV-1) belongs to the family of *Herpesviridae*, subfamily *Alphaherpesvirinae,* genus *Varicellovirus* [29] and causes several syndromes in the feline eye including conjunctivitis, epithelial keratitis, herpes stromal keratitis (HSK) and corneal sequestration [61, 84]. Despite the broad distribution of feline HSK, very little is known about its pathogenesis and no efficient therapy is currently available [36, 92]. There is strong experimental evidence for an immunopathological basis of the symptoms for HSK in cats. The disease is characterized by the infiltration of corneal stroma by inflammatory cells, later

on fibrosis and vascularization can lead to blindness. One hypothesis claims that persistent viral antigen gains access to the stroma during periods of protracted ulceration and is ineffectively cleared leading to immune-mediated tissue damage [65]. This is consistent with a recent study by Vögtlin and others, who studied the pathogenesis of HSK in cats [90]. In the first stage of ocular FeHV-1 infection, high virus titers as well as high amounts of viral DNA were present in ocular swabs of cats with HSK, whereas no antibodies against FeHV-1 were yet found in their serum. The second stage showed elevated viral DNA levels combined with negative or low titers of infectious virus. At the same time serum antibodies against FeHV-1 began to emerge. The third stage showed ever decreasing viral DNA levels, accompanied by negative virus isolation. Interestingly, keratitis occurred during the second stage of infection, when anti-viral antibody titers emerged, while the viral load seemed to continually decrease. Such a situation may be achieved by sustained proinflammatory signalling, probably through TLRs.

2.3. HSK models

In humans, herpes stromal keratitis (HSK) triggered by HSV-1 infection is the most common infectious cause of blindness in the western world and is considered to be the result of recurrent infection following reactivation of virus from the trigeminal ganglion [85]. Several models for human HSK have been established in mice [5, 20, 39, 45, 93]. Experiments using HSV-1 in those mouse models strongly support the hypothesis of an immunomediated pathogenesis [17]. Especially T cell induced inflammatory events, mainly orchestrated by CD4 positive cells, are thought to play an essential role [13, 30, 49]. Proinflammatory cytokine and chemokine production [16, 50], influx of innate immune cells, especially polymorphnuclear leukocytes (PMN) [27, 88, 94], and angiogenesis [12, 18, 51] represent the main factors which lead to the recruitment of these crucial cells and set the stage for the subsequent pathology. In the feline system, the origins, triggers, and molecular consequences

of these apparent inflammatory events are still poorly understood. Unfortunately, FeHV-1 does not replicate in mice. Therefore, mouse models could not be established for the feline variant of HSK.

2.4. Toll like receptors

A major topic of immunological research is the question how the immune system is able to differentiate between foreign antigens and self-antigens. New insights concerning this field of interest have been gained in the last few years [19, 26, 45]. The mechanisms underlying the recognition of the pathogens early in infection by the innate immune system are of special interest. So called pattern recognition receptors (PRRs) were found to play an important role in the sensing of invading infectious agents.

The Toll like receptors (TLRs) belong to the PRR class of molecules and are able to detect a broad range of pathogens [87]. First, bacteria and fungi were described to be recognized by TLRs. However, recent studies demonstrated the involvement of these receptors in viral infections [3, 15, 33, 42, 54, 75]. In addition, two genes of vaccinia virus were shown to influence Toll like receptor signaling indicating an important role of TLRs in antiviral defense [22, 40]. Distinct structures of infectious agents named pathogen-associated-molecular patterns (PAMPs) serve as recognition elements for the TLRs. Four types of PAMPs have been identified related to viruses including double stranded RNA, CpG DNA, single stranded RNA and envelope glycoprotein's (Table 1).

Interestingly, several members of the herpesvirus family including *alpha*-, *beta*- as well as *gammaherpesviruses* were shown to be detected by different Toll like receptors [19, 25, 38, 63, 80]. Unmethylated CpG DNA, which is present in herpesvirus genomes, depicts the PAMP for Toll like receptor 9 [14, 43]. The induction of inflammatory cytokines by the infection with various herpesviruses including HSV-1 [45, 53], HSV-2 [59] and murine Cytomegalovirus (MCMV) [52] was demonstrated and is supposed to rely on TLR 9.

Induction of antiviral immunity by TLRs in stromal and dendritic cells was demonstrated by Sato et al. [79]. Krug et al. [53] found that the Interferon response to HSV-1 depends on TLR 9 and MyD88, an adaptor molecule involved in TLR signaling. Hochrein et al. described a TLR dependent and independent induction of IFNγ by HSV-1 [45]. Similarly, Lund et al. [59] noted that HSV-2 induced production of IFNγ in mouse dendritic cells through a TLR 9 mediated pathway. In addition Tabeta and colleagues as well as Krug et al. described an essential role of TLR 9 in the immune defence against MCMV including cytokine responses and NK cell activation [52, 86]. TLR 9 is not the only member of the Toll like receptor family which plays a crucial role in the defense against herpesvirus infection. Jin et al. [47] described a TLR 7 upregulation in active and inactive HSK corneas and an implication of TLR 4, 8 and 9 in the pathogenesis of active HSV infections. Also Li et al. [57] demonstrated an expression of TLR 7 in human corneal epithelial cells. In another study Li et al. [56] showed that HSV-2 infection induces expression of TLR 7 and proinflammatory cytokines in cervical epithelial cells. TLR 2 as well as TLR 3 were found to be involved in cytokine signaling triggered by herpesviruses. Compton et al. showed that Toll like receptor 2 and CD14 recognize CMV virions and influence thereby cytokine release [26]. Kurt-Jones et al. demonstrated that TLR 2 mediates the inflammatory cytokine response to HSV-1 [55]. Ashkar et al. [11] published that TLR 3 agonist, and not TLR 4, protects against genital herpes infection in the absence of inflammation seen with CpG DNA, and Tohyama et al. [89] declared the MIP-1α production of normal human keratinocytes in response to HSV-2 infection via TLR 3. The importance of an intact TLR-system was shown by Bustamante et al. [24] and Sancho-Shimizu et al. [78] with the description of a TLR 3 mutation and UNC93B1 impair TLR 3 response that led to a predisposition to herpes simplex encephalitis. Böttcher et al. [20] stated that the herpes simplex encephalitis caused a rise of TLR 4 mRNA. Despite the remarkable research on Toll like receptors and their role in recognition of pathogens in general, not much is known about their distribution and function in cats and the various roles of TLRs have not yet been

addressed in the context of infections of cats with FeHV-1. Expression of Toll like receptors in feline lymphoid tissue [46] and cloning of feline Toll like receptor 4 [9] is published. Abujamra et al. showed an interaction between the Feline Leukemia virus and Toll like receptor 3 [1]. Overall, it seemed that TLR 2 (recognition of virions), TLR 3 (dsRNA), TLR 4 (LPS), TLR 7 (viral ssRNA) and TLR 9 (CpG DNA) might play an important role in the context of herpesvirus infections in cats.

2.5. Aims / Hypothesis

The aim of this study was to gain first insight into the interaction between Felid herpesvirus-1 (FeHV-1) and the feline innate immune system during the first stages of infection.

Based on experiments using HSV-1 and other herpes viruses [56, 57], we presumed a potential role of Toll like receptors in the pathogenesis of diseases associated with FeHV-1 infection in cats. If this were true, the induction of proinflammatory cytokines upon infection of cells with FeHV-1 could be expected. However, it may be that different types of cells react in different ways to the FeHV-1 challenge.

In order to test this hypothesis, the induction of proinflammatory cytokine mRNA by FeHV-1 infection as well as the influence of FeHV-1 infection on TLR mRNA expression was analyzed in Crandell Reese Feline Kidney (CRFK) cells as well as in feline peripheral blood mononuclear cells (PBMC). As positive controls for proinflammatory cytokine induction, the cells were treated with the TLR 4 ligand LPS and, in one series of experiments, with Gardiquimod, a synthetic TLR 7 ligand.

3. Materials and Methods

3.1. Cells

Crandell Reese Feline Kidney (CRFK) cells and feline peripheral blood mononuclear cells (PBMC) were maintained in Iscove's modified Dulbecco's medium (Sigma, Buchs, Switzerland) supplemented with 1 % L-Glutamine (2mM) (Sigma, Buchs, Switzerland), 1 % Non essential amino acids (NEAA) (Invitrogen, Basel, Switzerland), 10% fetal calf serum (FCS) (Omnilab, Mettmenstetten, Switzerland), 5 ml Antibiotic-Antimycotic (Invitrogen, Basel, Switzerland) and grown at 37°C.

3.2. Isolation of feline peripheral blood mononuclear cells

EDTA blood samples from clinically healthy blood donor cats were taken and used for the isolation of feline peripheral blood mononuclear cells (PBMCs). Briefly, 10ml of EDTA blood were mixed with 10ml of ice-cold Hank`s Buffered Salt Solution (HBSS), 1 x (-) CaCl2 (-) MgCl2 (Invitrogen, Basel, Switzerland) and 4ml of a FicollPaque™Plus (GE Healthcare, Zurich, Switzerland) were overlaid with 10ml of the blood/HBSS suspension. The sample was centrifuged at 800g, 4°C for 40 minutes and the ring containing the mononuclear cells was carefully removed and transferred into a new tube. Furthermore, the cells were washed twice with ice-cold HBSS in a total volume of 50ml. The cell pellet was suspended in cell culture medium and maintained as described above.

3.3. Stimulation of cells using LPS and Gardiquimod

In order to have a positive control for proinflammatory cytokine induction, PBMCs as well as CRFK cells were treated with 1µg/ml Lipopolysaccharide (LPS) (Sigma, Buchs, Switzerland). To examine the response of Toll like receptor 7, cells were stimulated with the synthetic Toll like receptor 7 ligand Gardiquimod (Invivogen, Toulouse, France) at a concentration of 5µg/ml.

3.4. Viral infections

For viral infection the Felid herpesvirus 1 (FeHV-1, isolate UT88; Pharmacia, UpJohn) as well as a recombinant of the FeHV-1 expressing the green fluorescent protein (GFP) were used [77]. Stocks of UT88 and recombinant FeHV-1 used in this study were propagated on CRFK cells grown as described before. The titer of virus stocks was determined by standard $TCID_{50}$ determination assay on CRFK cells using 96 well cell culture plates. Stocks were stored at -80°C in 1ml aliquots, and a fresh aliquot of stock virus was thawed and used for each experiment. As a negative control (mock) uninfected CRFK cell preparations were processed in the same way as virus stock preparations. To infect cells, either PBMCs or CRFK cell cultures were infected at low with 0,5 and high with 5 multiplicity of infection (moi = is the ratio of the number infections agents –virus- to infection targets –cell-) and grown for 1 hour at 37°C in medium containing 0% FCS. After 1 hour of adsorption, the inoculum was removed and cells were further cultivated at 37°C using medium including 2% FCS. At the indicated time, cells were processed for RNA preparation.

3.5. Real – Time quantitative Polymerase chain reaction

3.5.1. RNA extraction and Reverse transcription of isolated RNA into cDNA

After different time points cells were harvested and total cellular RNA was isolated using the RNAeasy Kit (QIAgen, Basel, Switzerland) according to the manufacturer's protocol with exception of DNAse digestion, which was performed for 30min instead of 15min. The extracted RNA was eluted in 60µl of elution buffer and concentrations as well as purity were determined using the NanoDrop^{R} ND 1000 spectrophotometer (Witec AG, Littau, Switzerland). Isolated RNA was reverse transcribed into cDNA with the Reverse Transcriptase Kit (Promega, Dübendorf, Switzerland) following the manufacturer's instruction. The reaction proceeded for 1 hour at 42°C and was then inactivated for 5 min at

95°C. The cDNA preparation was stored at -80°C and later used for quantitative Polymerase chain reaction analysis.

3.5.2. Quantitative Polymerase chain reaction

The reaction was performed on an iCycler IQ RealTime detection system (BioRad, Reinach, Switzerland). The cDNA was amplified by PCR using the specific primers listed in Table 2. PCR amplification was carried out in 20µl reactions containing (final concentration) 10µl iQ SYBR Green Supermix (BioRad), 1µl (400nM) of each primer, 4µl of cDNA (30ng) and 5µl of sterile DEPC treated water. The conditions were set as follows: 10min 95°C, followed by 45 cycles consisting of 15sec 95°C denaturation and 1min 60°C annealing-elongation. In addition a melt curve analysis was performed. Real-time data were collected and analyzed using the iCycler IQ Real-time Detection System Software V.3.0 (BioRad, Hercules, CA). Threshold cycle numbers (Ct) were transformed using the delta delta Ct methods as outlined in ‘‘User Bulletin 2’’ provided by Applied Biosystems using GAPDH as the reference housekeeping gene.

Total RNA from non-infected CRFK cells was isolated and reverse transcribed as described above. The amplification products for TLR 2, 3, 7, 9 and GAPDH from Real-Time PCR were subjected on a 2% agarose gel containing ethidiumbromide and analyzed under UV light.

4. Results

4.1. Feline peripheral blood mononuclear cells are susceptible to FeHV-1 infection

In order to evaluate the potential of Felid herpesvirus 1 to infect feline peripheral blood mononuclear cells, a recombinant of FeHV-1 strain UT 88 expressing the green fluorescent protein (GFP) [77] was used. CRFK cells, which are known to be susceptible to FeHV-1 infection, were used as a positive control. Feline peripheral blood mononuclear cells (PBMC's) and CRFK were infected at a multiplicity of infection of 5 and microscopically examined for expression of GFP 24 hours post infection. For this the cultures were analyzed under an epifluorescence microscope (Axiovert S 100, Carl Zeiss AG, Feldbach Switzerland) using ultraviolet and a filter for GFP [77]. In both cell types green fluorescence was detected indicating a successful infection (Fig.1 A and B), whereas the efficiency of infection is much higher in the CRFK cells as in the PBMC`s.

4.2. FeHV-1 infection leads to proinflammatory cytokine induction

To gain a more detailed picture of the first stadium of infection with FeHV-1 and its consequence on innate immune responses, the induction of proinflammatory cytokine mRNA was measured. As a positive control, the expression of the proinflammatory cytokine Interleukin-6 (IL-6) mRNA was determined using feline PBMCs treated with Lipopolysaccharide (LPS). Indeed, LPS treatment strongly induced IL-6 mRNA synthesis in PBMCs at 24h post stimulation. The results from unstimulated and LPS-stimulated, respectively, PBMCs of three individual cats are shown in the Figure (Fig.2 A). Furthermore, CRFK cells were treated with LPS in order to evaluate their potential over 4h to produce a proinflammatory cytokine response. As shown in Figure 2B the ability of CRFK cells to produce IL-6 mRNA upon LPS stimulation was clearly demonstrated. In a second step, feline PBMCs and CRFK cells were infected with the wild-type FeHV-1 strain UT88 using a moi of

5. At different time points post infection, cells were harvested and analyzed for IL-6 mRNA expression by quantitative RT-PCR. An up-regulation of IL-6 mRNA level was found in PBMCs (Fig. 3A) as well as in CRFK cells (Fig. 3B) which were infected at different moi and assessed 24 hours post infection. Furthermore, the induction of the two other proinflammatory cytokine mRNAs - Interleukin-1 (IL-1) and Tumor necrosis factor alpha (TNFα) - was demonstrated (Fig. 4A and B).

Taken together, these results clearly indicate the ability of FeHV-1 to induce a proinflammatory cytokine response *in vitro*.

4.3. Expression of Toll like receptor mRNAs in non-infected PBMCs and CRFK cells

To form functional Toll like receptors, TLR-specific mRNAs must first be expressed in a given cell. Therefore, in a first step the mRNA expression of feline TLRs in non-infected PBMCs and CRFK cells was analyzed. Since several TLRs, including 2, 3, 7 and 9 have been suggested to be involved in the recognition of HSV-1 infection or other viral infections, these specific TLRs were focused on. The expression of mRNAs for all these receptors in both PBMCs (data not shown) as well as in CRFK (Fig.5) was determined by RT-PCR. As an internal control for RNA extraction and RT-PCR, the expression of the house-keeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used.

4.4. Expression of Toll like receptor mRNAs in FeHV-1 infected CRFK cells

Having shown the presence of TLR 2, 3, 7 and 9 mRNA in feline cells, the influence of FeHV-1 infection on the expression of these TLRs was studied. Therefore, CRFK cells were either infected or mock treated and after various time points the TLR mRNA levels were determined using qRT-PCR. Experiments concerning the expression of TLR 2, 3 and 7 mRNA were consistent and are shown in Figure 6. Interestingly, TLR 7 mRNA was up-regulated, visible in Figure 6C 12 hours post infection and in Figure 6D 36 hours post

infection, whereas TLR 2 and TLR 3 mRNA expressions were down-regulated, visible 5 hours post infection. In contrast, results regarding the level of TLR 9 mRNA expression following FeHV-1 infection were not consistent. In some experiments an up-regulation of TLR 9 mRNA was detected in others a down-regulation was observed (data not shown).

4.5. Stimulation of FeHV-1 infected CRFK cells with the TLR 7 agonist Gardiquimod

Having demonstrated an up-regulation of Toll like receptor 7 mRNA in FeHV-1 infected CRFK cells, the ability of infected and non-infected cells to respond to the TLR 7 agonist Gardiquimod, which mimics double-stranded RNA, was further investigated. Since a tremendous up-regulation of TLR 7 mRNA level was shown at 36 hours post infection, infected and mock treated cells were stimulated with Gardiquimod at this time point. As readout for TLR 7 stimulation IL-1 (Fig. 7A) as well as TNFα (Fig. 7B) mRNA expression were measured at 0, 4, 6 and 8 hours post stimulation. FeHV-1 infected cells were shown to produce significantly higher levels of proinflammatory cytokine mRNAs in response to Gardiquimod compared to mock treated cells. In Figure 7C the course of TLR 7 mRNA at 0, 4, 6, and 8 hours post stimulation with Gardiquimod is shown.

4.6. Stimulation of FeHV-1 infected PBMCs with the TLR 7 agonist Gardiquimod

After having shown a higher proinflammatory cytokine response to the TLR 7 agonist Gardiquimod of FeHV-1 infected CRFK cells compared to mock treated cells the reaction of FeHV-1 infected PBMCs to Gardiquimod was examined. As described in the previous section, FeHV-1 infected and mock treated PBMCs were stimulated with Gardiquimod 36 hours post infection. In contrast to the experiments with CRFK cells, the infection was performed using either moi 0.5 (Fig. 8A - 8C) or moi 5 (Fig. 8D – 8F). As readout of TLR 7 stimulation IL-1 (Fig. 8A, 8D) as well as TNF α (Fig. 8B, 8E) mRNA expression were measured 0, 4, 6 and 8 hours post stimulation with Gardiquimod. In addition, the mRNA

levels of TLR 7 (Fig. 8C, 8F) were measured at 0, 4, 6 and 8 hours post stimulation. In none of the experiments a significant difference between mock and FeHV-1 infected PBMCs with respect to cytokine production following Gardiquimod stimulation was observed.

5. Discussion

The interaction of FeHV-1 with its host, the cat, is poorly understood, particularly in the initial stage of infection. The aim of our study was to gain first insights into the interaction of FeHV-1 with the feline innate immune system. The prerequisite of these experiments was the availability of susceptible cells, which are able to elicit a proinflammatory cytokine response. First, we demonstrated that feline PBMC`s are susceptible to FeHV-1 infection using a recombinant FeHV-1 virus expressing the green fluorescent protein (GFP) [77]. This result is in accordance with a report by Palmer et al. [67], who described the infection of human PBMC's with HSV-1 and the following induction of IFN-α and IFN-β mRNA in these cells. In addition, we showed evidence for the induction of proinflammatory cytokines in feline PBMC's and CRFK cells 24 hours post infection with FeHV-1, i.e a significant up-regulation of IL-6 as well as the induction of two other proinflammatory cytokines, IL-1 and TNFα, which were shown at various time points post infection. The up-regulation of the cytokine mRNAs reached a maximum between 24 and 36 hours post infection.

Our results are in accordance with the finding of enhanced nasal cytokine transcription in FeHV-1 infected cats [48]. In addition experiments using Herpes simplex virus type 1 showed the induction of proinflammatory cytokines *in vitro* [57]. Aravalli et al. [7] demonstrated proinflammatory cytokine and chemokine production by murine microglial cells in response to HSV-1, as well as increased TNF-α and IL-6 mRNA through HSV-1 infection. Interestingly, Hayashi et al. [41] showed in their experiments an IL-6 release from infected corneal cells in humans.

Toll like receptors (TLRs) are involved in cytokine production in response to viral infections [26, 45, 52, 53, 58, 63, 81, 91]. We were able to demonstrate the expression of TLR 2, 3, 7 and 9 in non-infected CRFK cells and feline PBMC's. So far, TLR 2, 3, 7 and 9 were shown to be involved in the recognition of Herpes simplex virus 1 [4, 10, 32, 39, 70, 77]. Furthermore, we examined a potential influence of FeHV-1 infection on the expression of

feline Toll like receptors. Interestingly, we could show that infection of CRFK cells with FeHV-1 did not have the same effect on various members of the TLR family. TLR 2 as well as TLR 3 were shown to be down-regulated with a maximum of down-regulation at 5 hours post infection. In contrast, we found evidence that the expression of TLR 7 was up-regulated with a maximum at 36 hours post infection. These results are in accordance with a study using HSV-1 infected human corneal epithelial cells, in which a down-regulation of TLR 3 as well as an induction of TLR 7 could be demonstrated [57]. Jin et al. [47] described that all TLRs expressed diversely in the healthy cornea. TLR 4, 8 and 9 may be implicated in the pathogenesis of active HSV-1 infection in the cornea, whereas TLR 7 may play a key role in HSK whether it is active or not, since it was the only TLR which was up-regulated during the phase of virus persistence in ganglia without clinical symptoms in the eye. TLR 2, 3, 4, 5, 6, 8, 9 and 10 were down-regulated to this time.

Interestingly, the results for TLR 9 expression in our experimental set up were contradictory in each performed experiment. In contrast, earlier results described a TLR 2, 3 and TLR 9 up-regulation during HSV-1 infections in humans [93].

One possible explanation for this phenomenon, showing results contradictory to the published literature, could be based on the cell types used for the experiments [57]. It would be of interest to repeat the experiments using our experimental set up with feline corneal cells to address the question, whether or not it is the used cell type which leads to the different results regarding TLR 2, 3 and 9 expressions.

From the described results, we conclude that the infection with FeHV-1 appears to have a different impact on TLR expression depending on the type of receptor. Since TLR 7 was shown to be up-regulated by the infection with FeHV-1, we were interested in the reactivity of infected cells to a specific TLR 7 agonist. In order to address this question, we infected CRFK cells and treated them 36 hours post infection with the specific TLR 7 ligand Gardiquimod. The expression of IL-1 and TNFα following stimulation with Gardiquimod was

higher in FeHV-1 infected cells compared to mock treated cells. This result leads to the conclusion that FeHV-1 infection leads to an up-regulation of TLR 7 and as a consequence to an increased responsiveness of infected cells to the TLR 7 agonist Gardiquimod.

In contrast to the experiments using CRFK cells, such an effect could not be demonstrated in feline PBMCs. The induction of proinflammatory cytokines in FeHV-1 infected and mock treated PBMCs following stimulation with the TLR 7 agonist Gardiquimod did not differ.

A possible explanation for this can be the different infection level between CRFK cells and PBMC`s as demonstrated in Figure 1A and 1B. Using the recombinant eGFP expressing FeHV-1 the number of fluorescent cells was higher in cultured CRFK cells than in freshly isolated PBMCs. Therefore, the number of cells contributing to the effect is lower in the case of PBMC`s. However, the effect could also be based on the different cell type. To further study the effect of FeHV-1 infection on PBMCs, it would be valuable to separate the infected from the non-infected PBMCs by FACS in order to look at homogenous cell populations.

In summary, we demonstrate in this work the induction of proinflammatory cytokine mRNA by FeHV-1 infection in CRFK cells as well as in feline PBMCs. In addition, up-regulated mRNA expression and activity of Toll like receptor 7 in FeHV-1 infected CRFK cells were shown.

The experiments described in this study show an influence on proinflammatory cytokine and TLR expression in FeHV-1 disease. Furthermore, having more knowledge about TLR expression and function in the cat will open the door for future investigations of pathogen-host interactions in feline virology. Moreover, detailed studies about TLR induced signaling by FeHV-1 could lead to the development of novel therapeutic strategies [5, 21, 23, 44].

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7. Figure legends, Tables and Figures

Figure 1

Infection of feline cells with a recombinant FeHV-1 expressing eGFP. Feline PBMCs **(A)** and CRFK cells **(B)** were infected with moi 5 and microscopic pictures were taken 24 hrs post infection.

Figure 2

IL-6 mRNA expression of cells treated with 1µg/ml LPS measured by qRT-PCR. **(A)** IL-6 mRNA levels in PBMCs isolated from three individual cats at 24h after mock-treatment or LPS-stimulation. **(B)** IL-6 mRNA levels **at** 0, 2, and 4h after mock- or LPS-stimulation in CRFK cells. The Error Bars of **(B)** were calculated on the basis of 3 experiments.

Figure 3

IL-6 mRNA expression of wt FeHV-1 infected cells measured by qRT-PCR. PBMCs **(A)** or CRFK cells **(B)** were infected at different moi and IL-6 mRNA levels were assessed 24h post infection. The Error Bars of **(B)** were calculated on the basis of 3 experiments.

Figure 4

Proinflammatory cytokine expression in wt FeHV-1 infected (moi 5) CRFK cells measured by qRT-PCR. IL-1 **(A)** and TNFα **(B)** mRNA expression of CRFK cells 0, 6, 12, 24, and 36h post infection was determined. The Error Bars here were calculated on the basis of 3 experiments.

Figure 5

Agarose gel electrophoresis of Toll like receptor specific RT-PCR amplimers in non-infected CRFK cells.

Figure 6

Toll like receptor expression in wt FeHV-1infected CRFK cells measured by qRT-PCR. CRFK cells were infected with wt FeHV-1 at moi 5 and TLR 2 **(A)**, TLR 3 **(B)** and TLR 7 **(C, D)** mRNA expression were assessed at different time points post infection. The Error Bars of (**A**) and (**B**) were calculated on the basis of 4 experiments and (**C**) and (**D**) on 3 experiments.

Figure 7

Proinflammatory cytokine and TLR 7 mRNA expression in CRFK cells treated with Gardiquimod. Mock or with wt FeHV-1 (moi 5) infected CRFK cells were stimulated with 5µg/ml Gardiquimod 36h post infection. 0, 4, 6, 8h post stimulation IL-1 **(A)**, TNF **(B)** as well as TLR 7 **(C)** mRNA expression was determined. The Error Bars here were calculated on the basis of 3 experiments.

Figure 8

Proinflammatory cytokine and TLR 7 mRNA expression in PBMCs treated with Gardiquimod. Mock or with wt FeHV-1, moi 0,5 (**A**),(**B**) and (**C**) and moi 5 (**D**), (**E**) and (**F**), infected PBMCs were stimulated with 5µg/ml Gardiquimod 36h post infection. 0, 4, 6, 8h post stimulation IL-1 **(A)** and **(D)**, TNF **(B)** and **(E)** as well as TLR 7 **(C)** and **(F)** mRNA expression was determined. The Error Bars here were calculated on the basis of 3 experiments.

Table 1. Toll like receptors and their Pathogen Associated Molecular Patterns (PAMPs)

Toll like receptor	PAMP	Agonist	Pathogen
TLR 2	Peptidoglycan, lipopeptides, atypical LPS (bacteria)		Measles virus hemagglutinin protein Herpes simplex virus (HSV-1) Human cytomegalovirus
	Zymosan, phospholipomannan (fungi)		
	GPI anchor (protozoa)		
	Envelope protein (virus)		
TLR 3	Poly (I:C), dsRNA (virus)		Poly (I:C) Murine cytomegalovirus (MCMV) Vesicular stomatitis virus (VSV) Lymphocytic choriomeningitis virus Reovirus West Nile virus
TLR 4	LPS (bacteria)		Mouse mammary tumor virus envelope protein Respiratory syncytial virus
	Mannan, glucuronoxylomannan (fungi)		
	Glycoinositolphospholipids (protozoa)		
	RSV fusion protein (virus)		
TLR 7 / TLR 8	Synthetic imidazoquinoline-like molecules, ssRNA (virus)	Gardiquimod	R848 Imiquimod Loxoribine Human immunodeficiency virus VSV Influenza virus Feline papillomavirus
TLR 9	GpG DNA (bacteria, protozoa, virus), dsDNA		HSV-1 HSV-2 MCMV Synthetic CpG DNA
	Hemozoin (protozoa)		

(State-of-the-art of Immunology)

Table 2. Feline specific primer sequences for Real-time PCR

Primer name	**Sequence in 5'-3' orientation**	**Reference**
GAPDH forward	GCCGTGGAATTTGCCGT	[35]
GAPDH reverse	GCCATCAATGACCCCTTCAT	[35]
IL-1 forward	AATGACCTGTTCTTTGAGGCTGAT	[35]
IL-1 reverse	CCAGAAAACTGTGGCTCAGGTT	[35]
IL-6 forward	CCCTGCAGACAAAATGGAAGA	[35]
IL-6 reverse	GTGCCTCCTTGCTGTCCTCA	[35]
TNF α forward	CTTCTCGAACTCCGAGTGACAAG	[35]
TNF α reverse	CCACTGGAGTTGCCCTTCA	[35]
TLR 2 forward	AGACTCTACCAGATGCCTCCTTCT	[46]
TLR 2 reverse	GCGTGAAAGACAGGAATTCACAGG	[46]
TLR 3 forward	CAACAACTTAGCACGGCTATGG	GenBank Accession Nr. DQ266436 (Felis catus Toll-like receptor 3 (TLR3) mRNA, complete cds)
TLR 3 reverse	AATGTGGAGGTGAGAAAGACCC	GenBank Accession Nr. DQ266436 (Felis catus Toll-like receptor 3 (TLR3) mRNA, complete cds)
TLR 7 forward	AATCGGTTTCTGTGCACCTGT	GenBank Accession Nr. DQ333223 (Felis catus Toll-like receptor 7 (TLR7) mRNA, complete cds)
TLR 7 reverse	GCCAAGTAAGGAATAGTCACCTCTG	GenBank Accession Nr. DQ333223 (Felis catus Toll-like receptor 7 (TLR7) mRNA, complete cds)
TLR 9 forward	GGACCTGAGTGAGAACTTCCTATATGAC	GenBank Accession Nr. AY859724 (Felis catus Toll-like receptor 9 (TLR9) mRNA, complete cds)
TLR 9 reverse	GGTAATTGAAAGACAAGTTGAGTCTGC	GenBank Accession Nr. AY859724 (Felis catus Toll-like receptor 9 (TLR9) mRNA, complete cds)

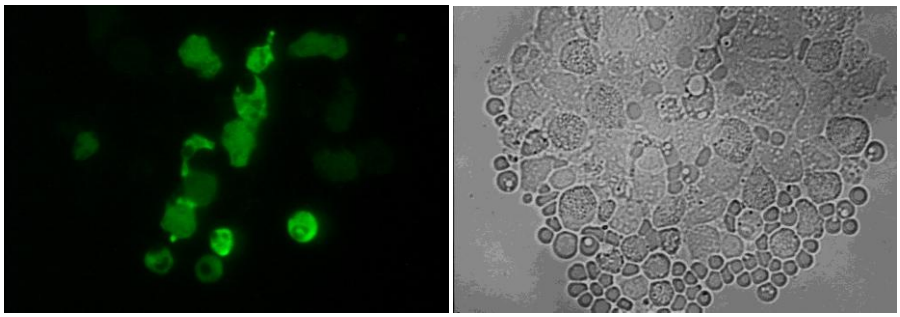


Figure 1 A

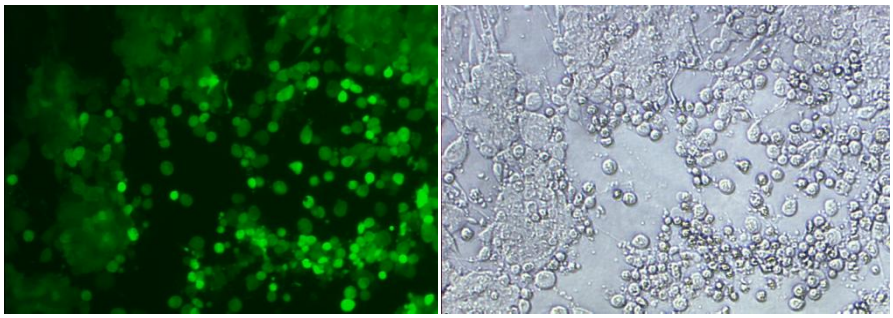


Figure 1 B

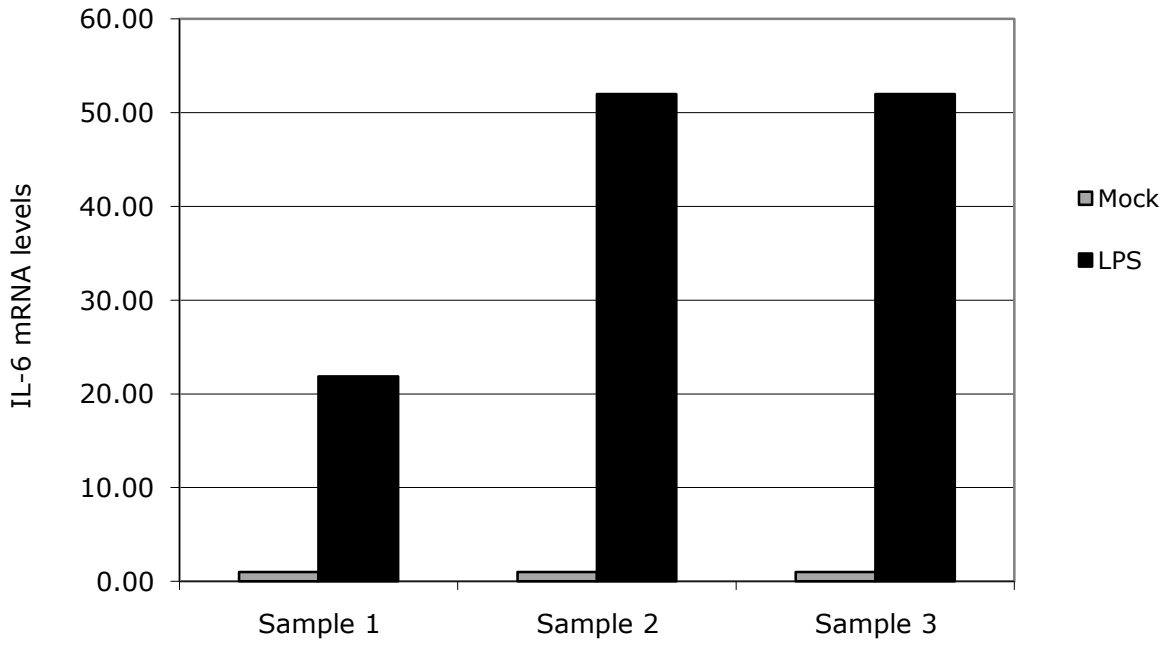
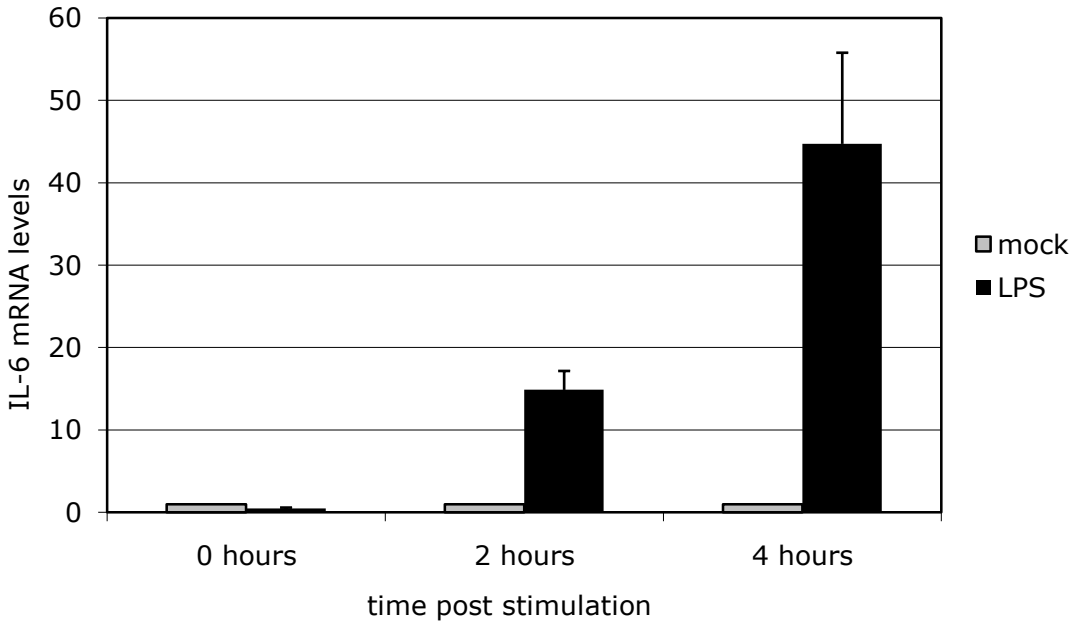


Figure 2A



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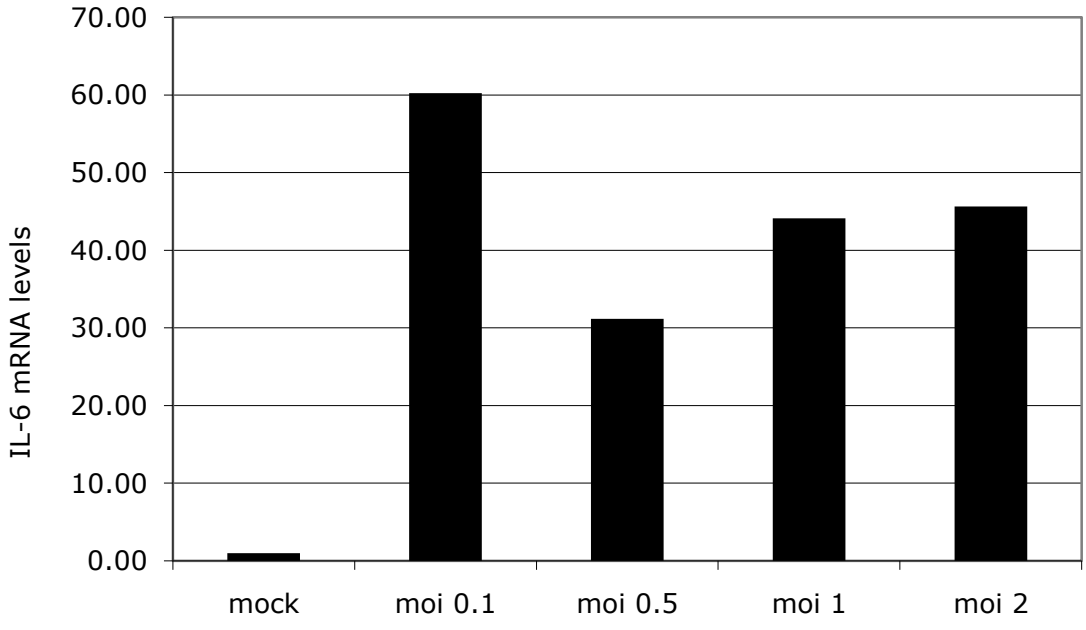

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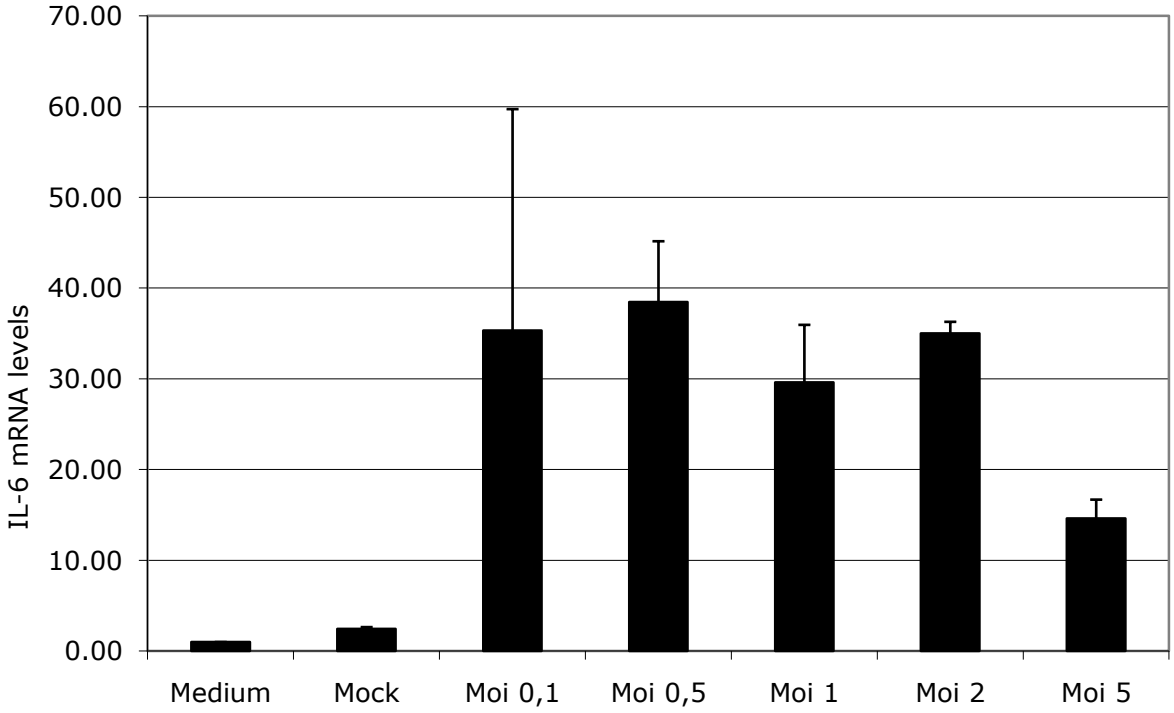
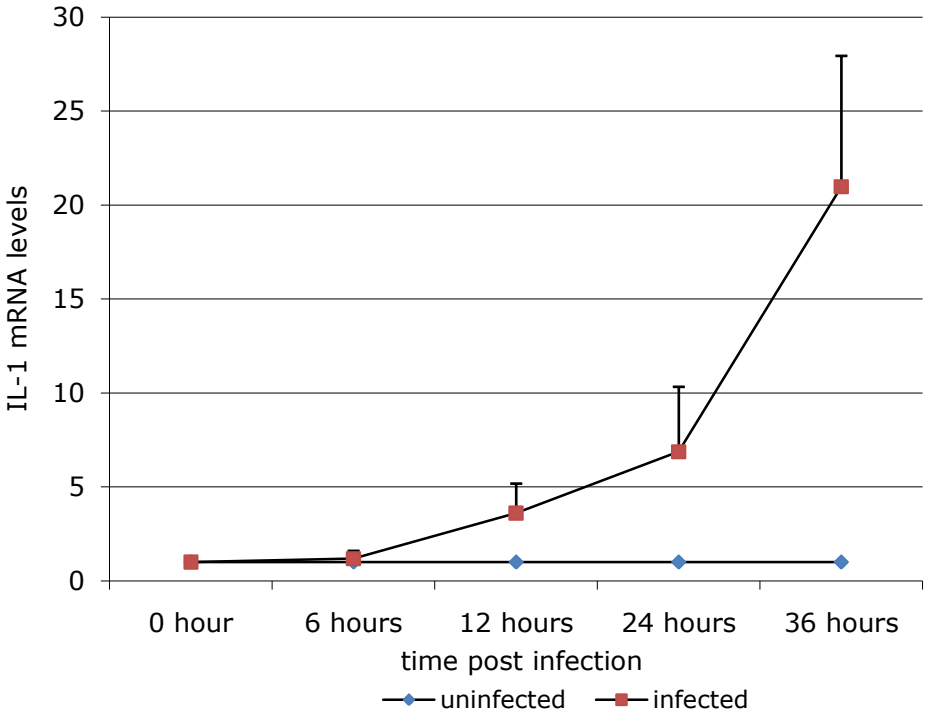


Figure 3 B



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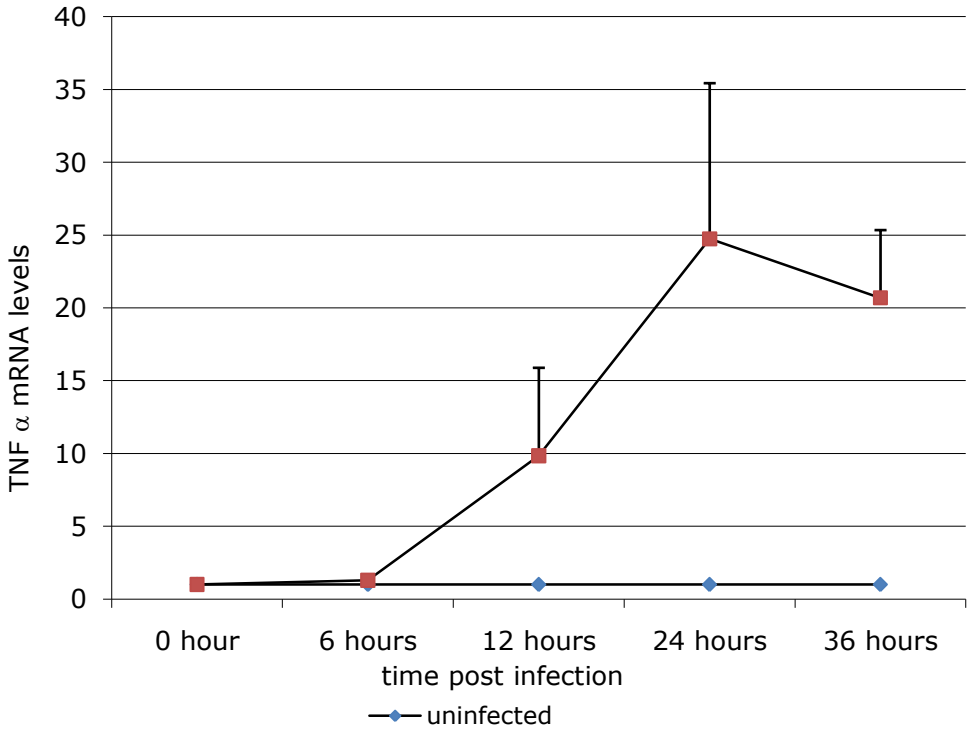

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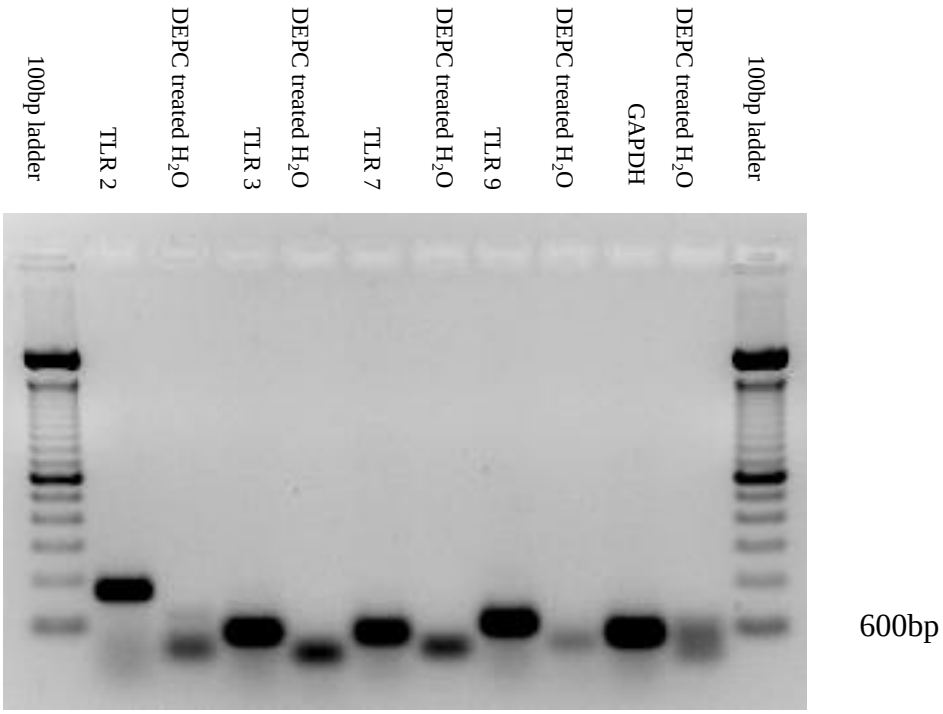

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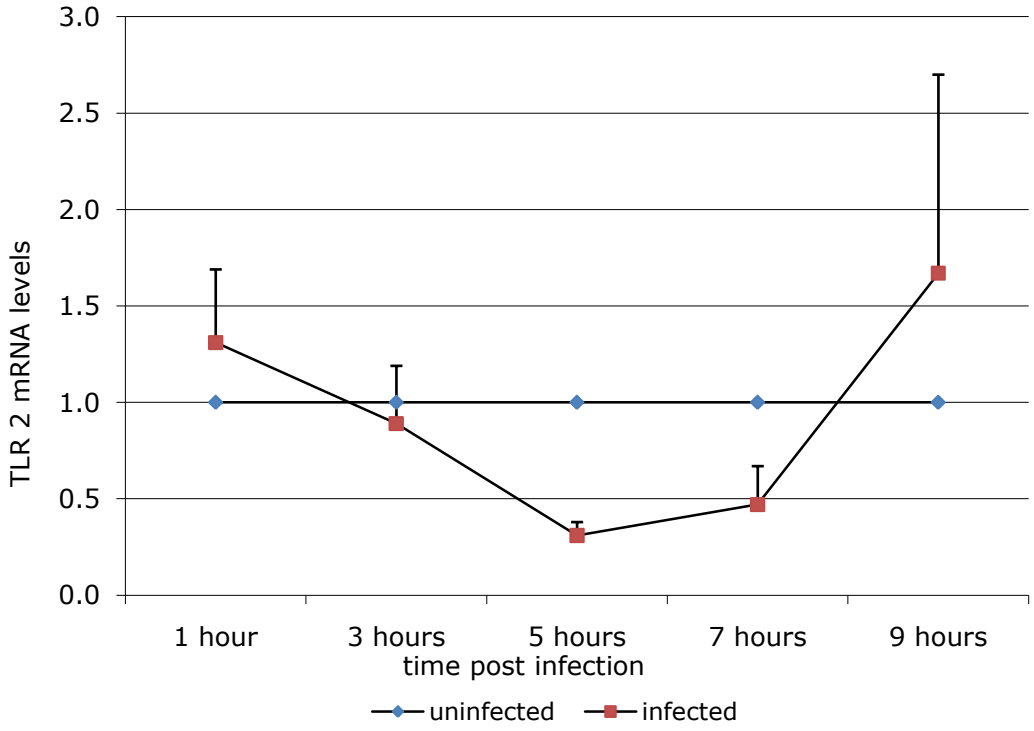

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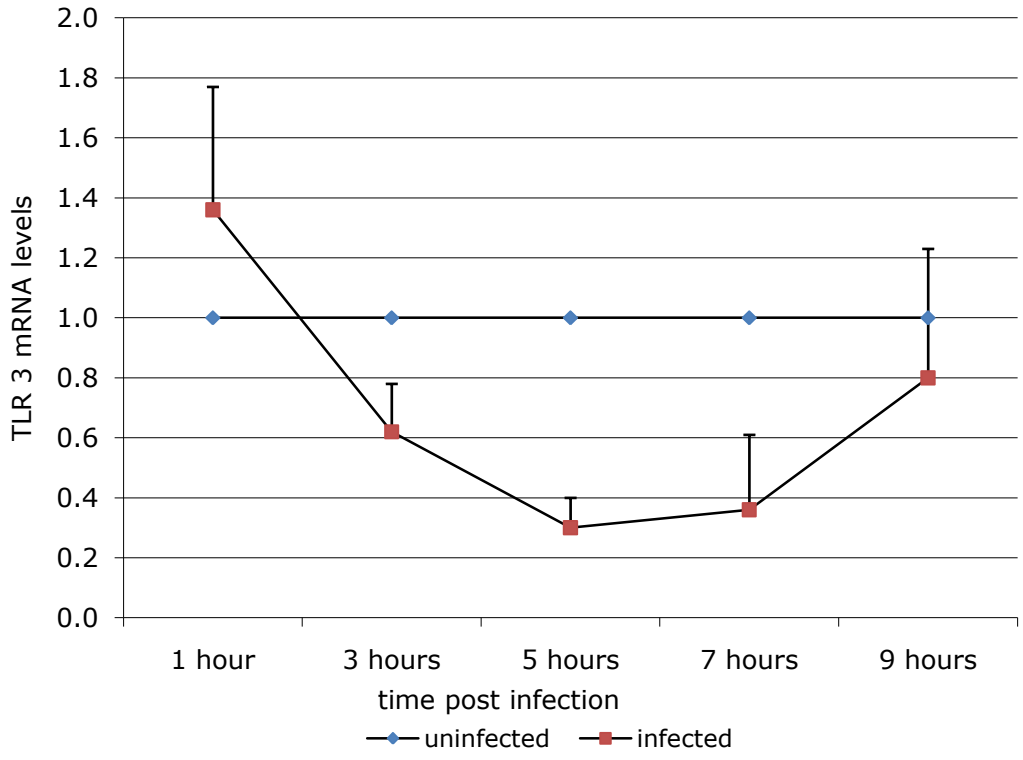

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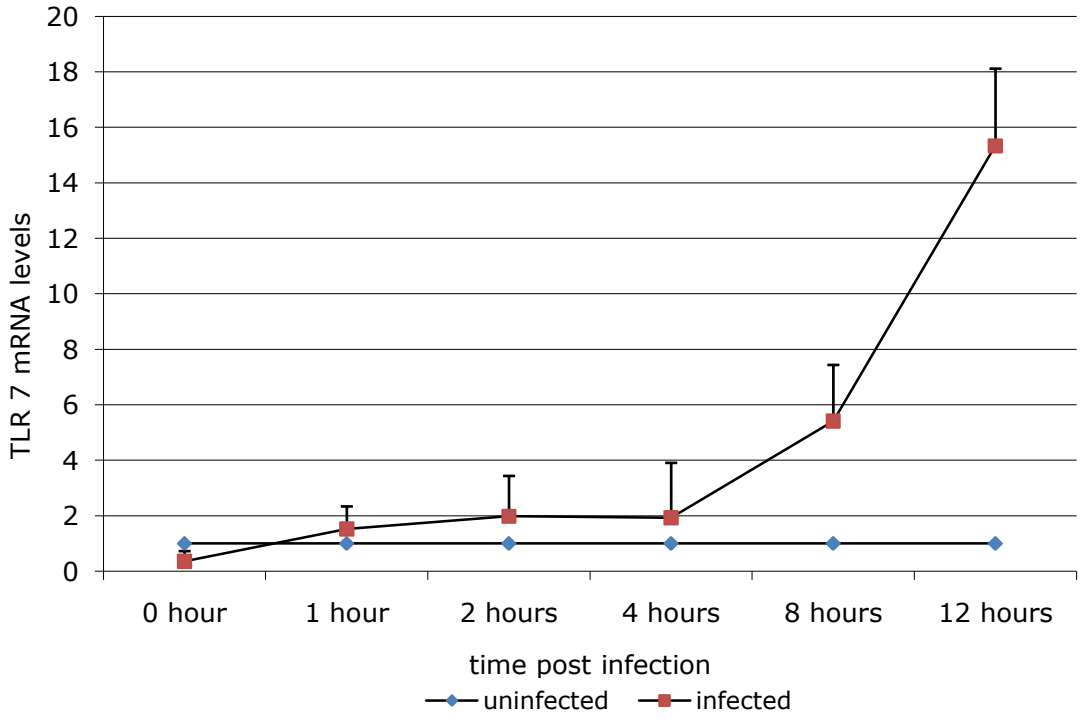


Figure 6 C

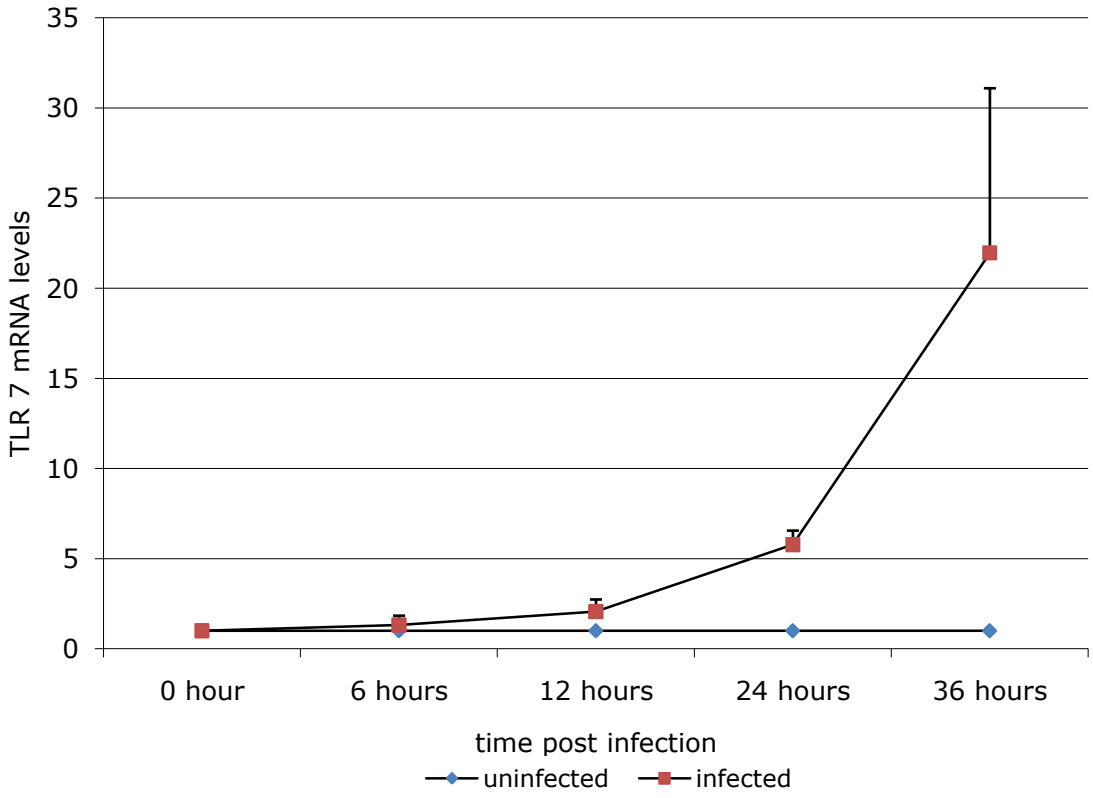


Figure 6 D

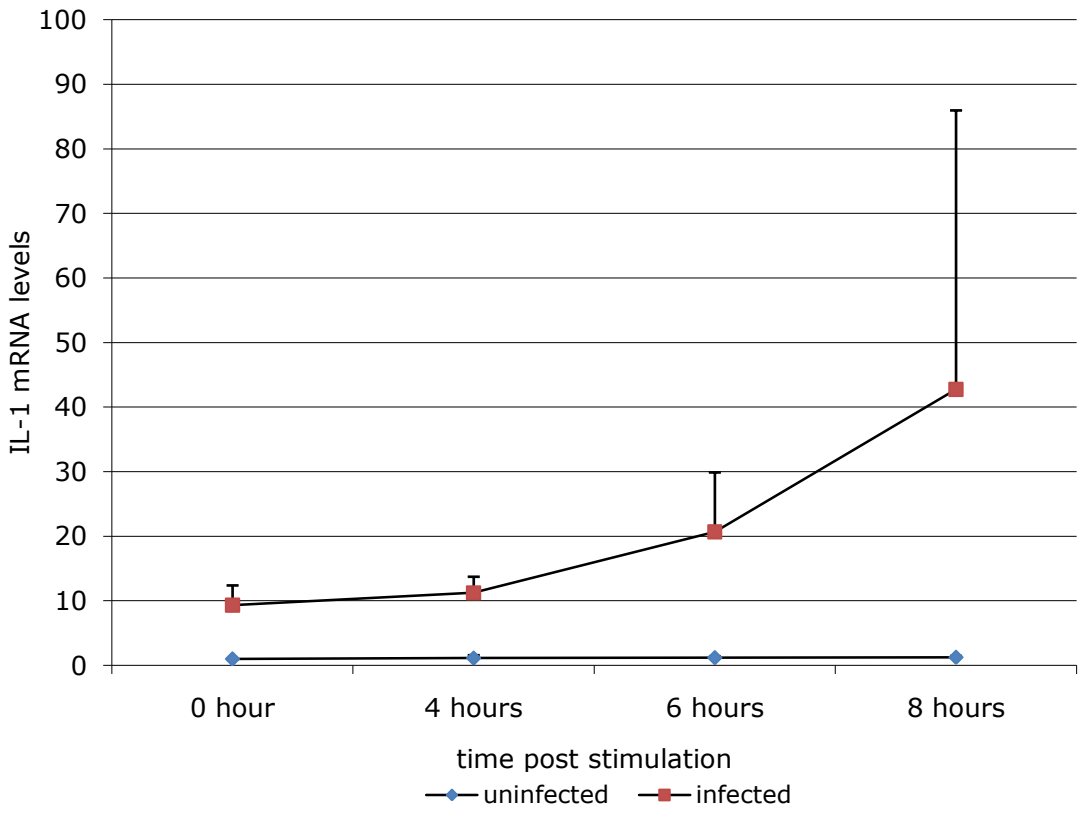
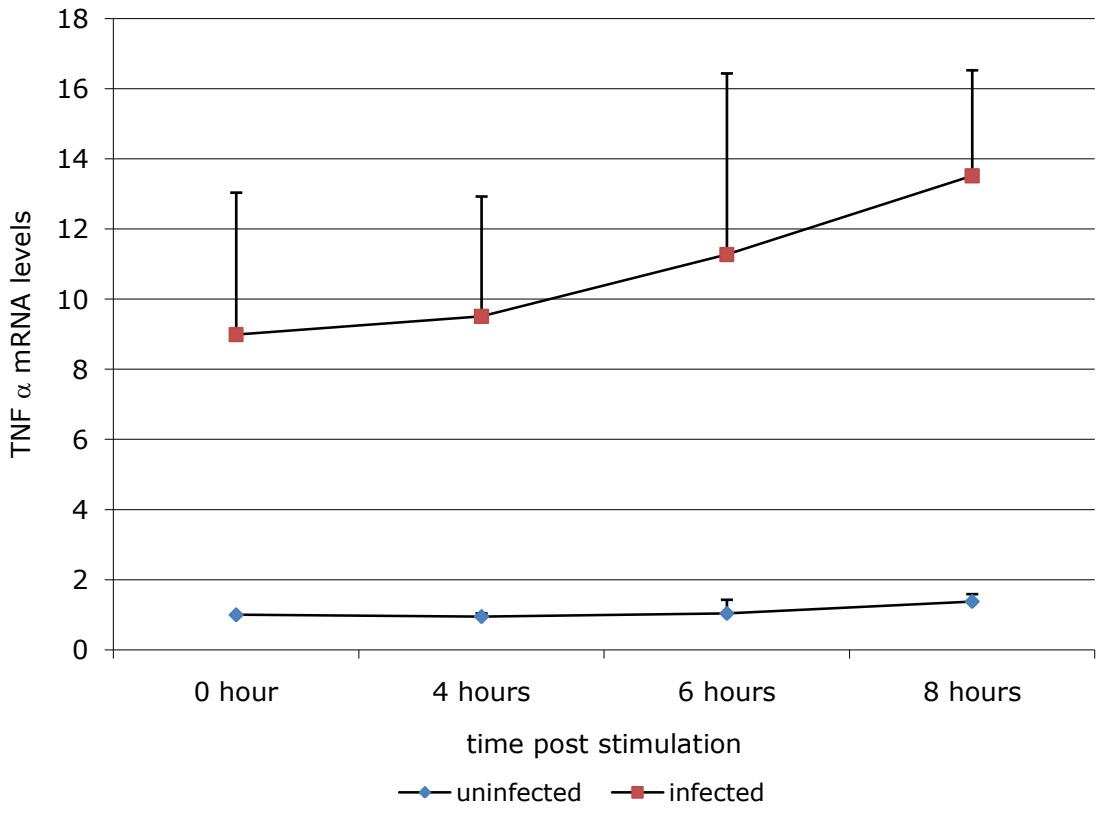


Figure 7 A



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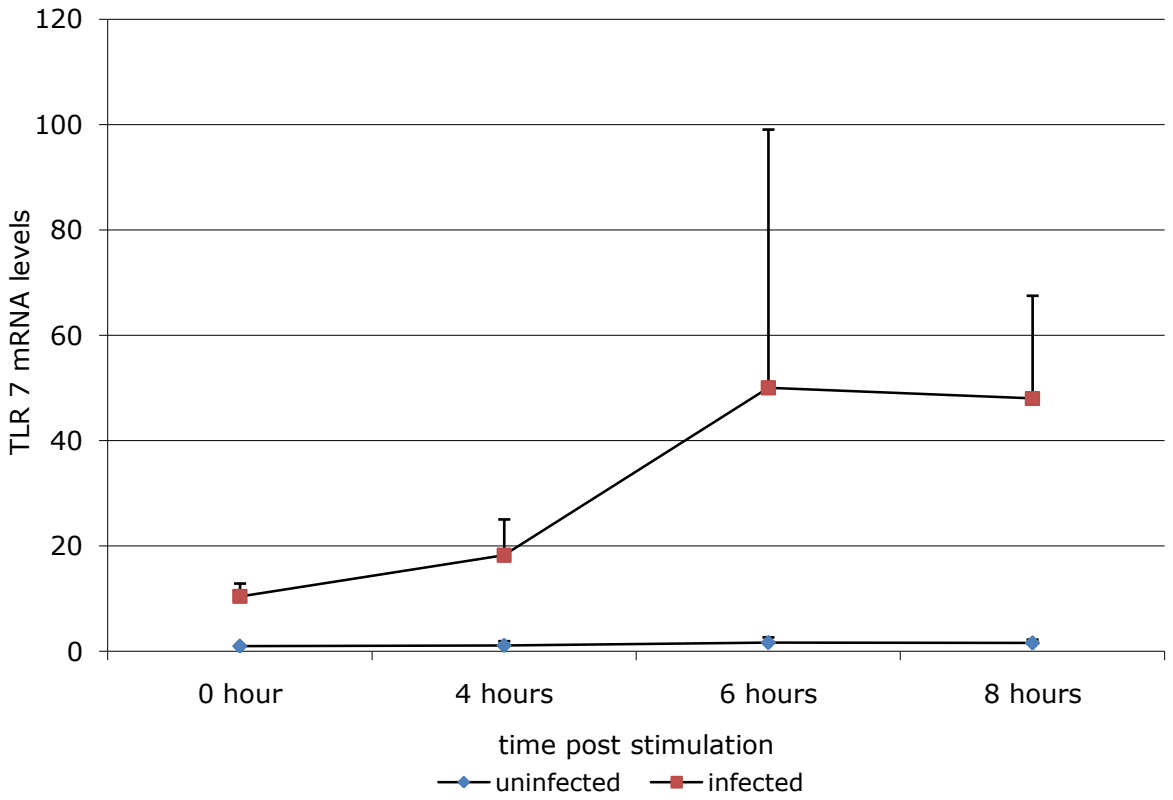

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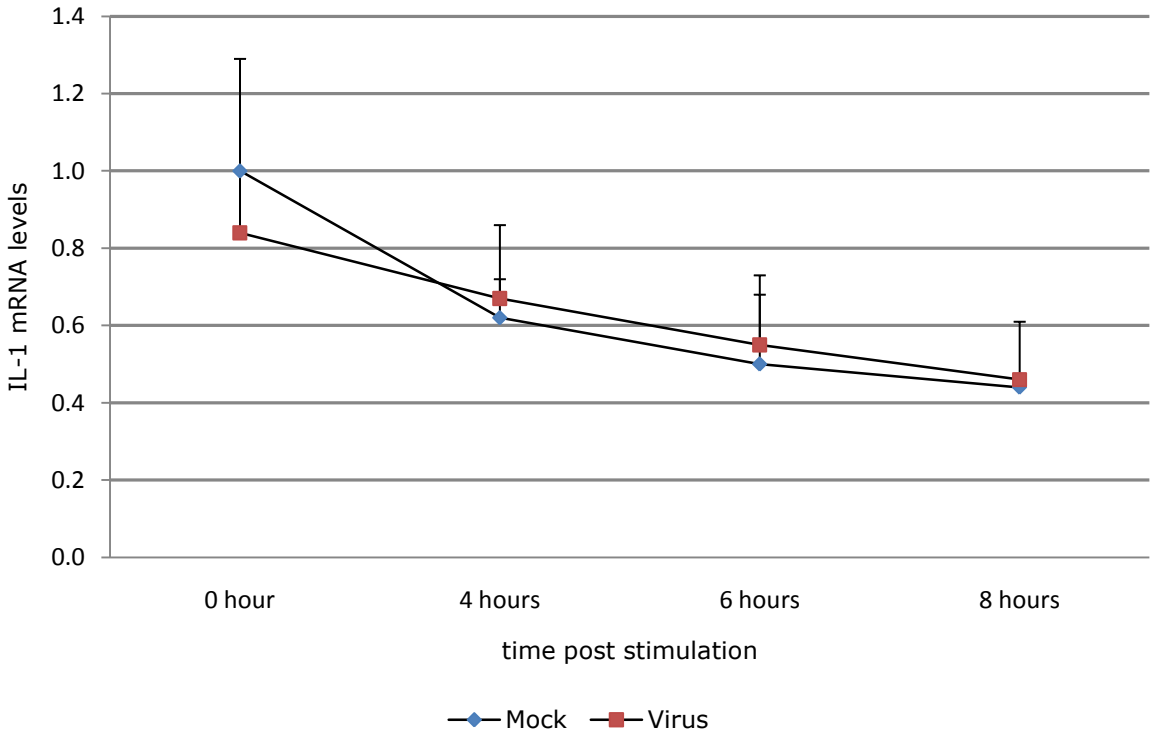
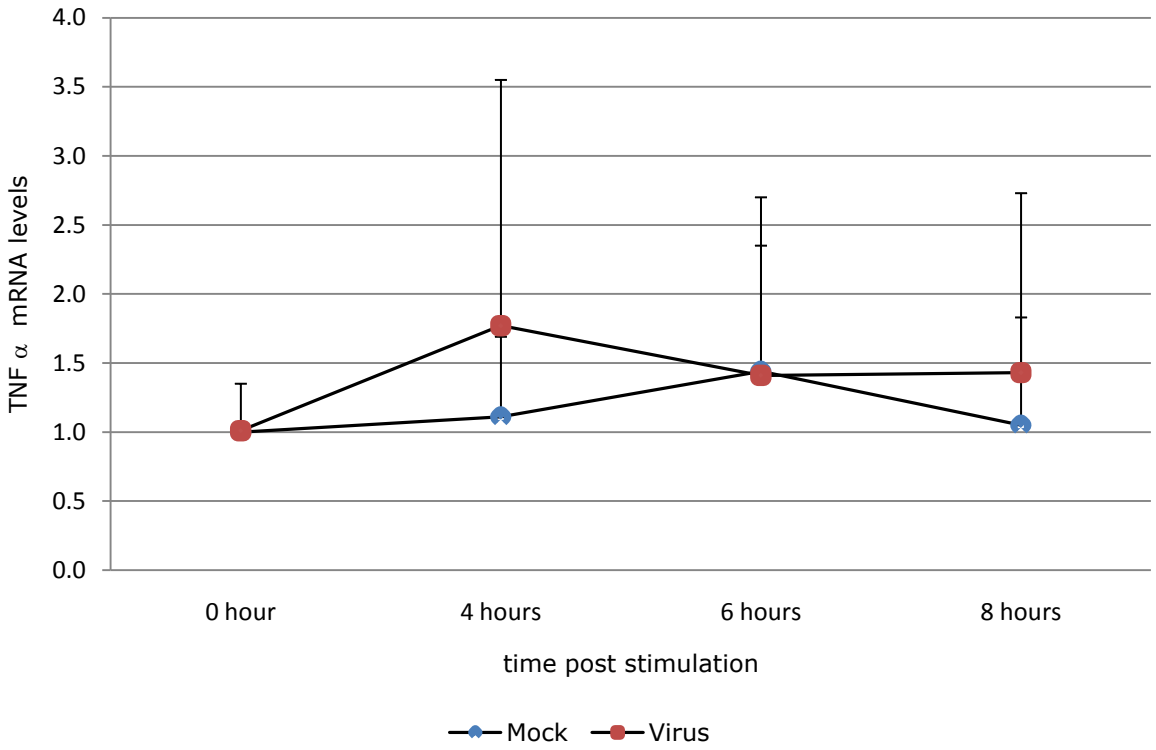


Figure 8 A



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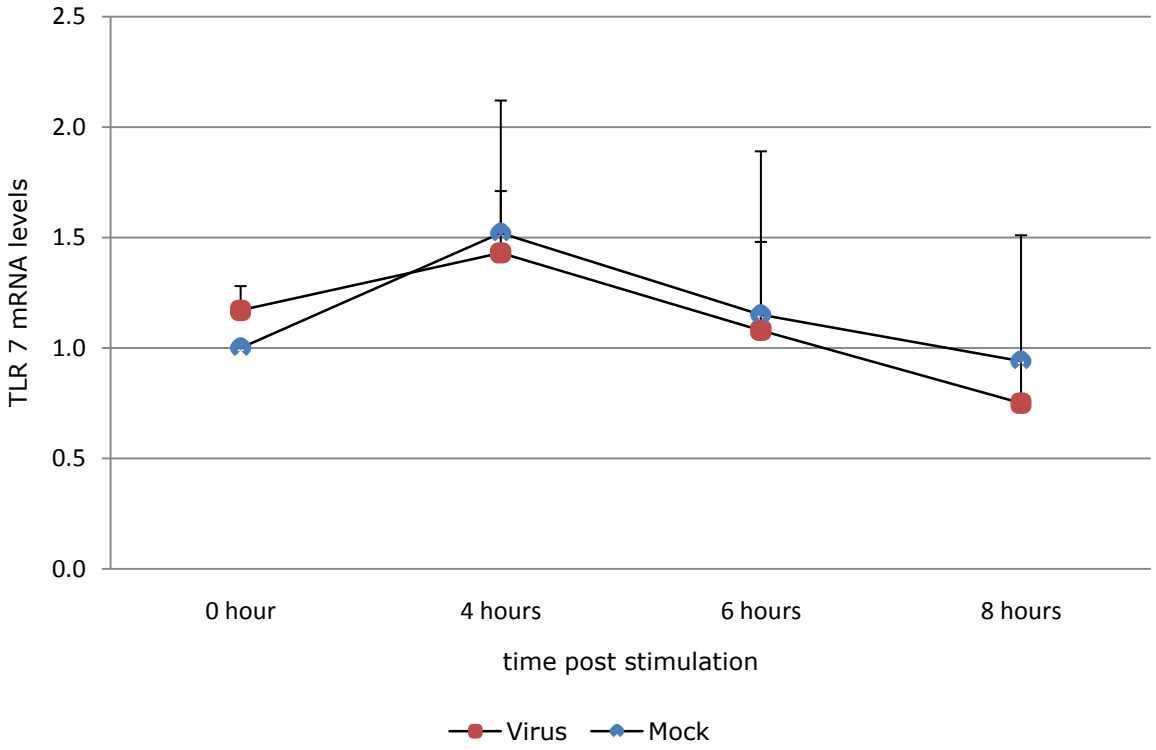

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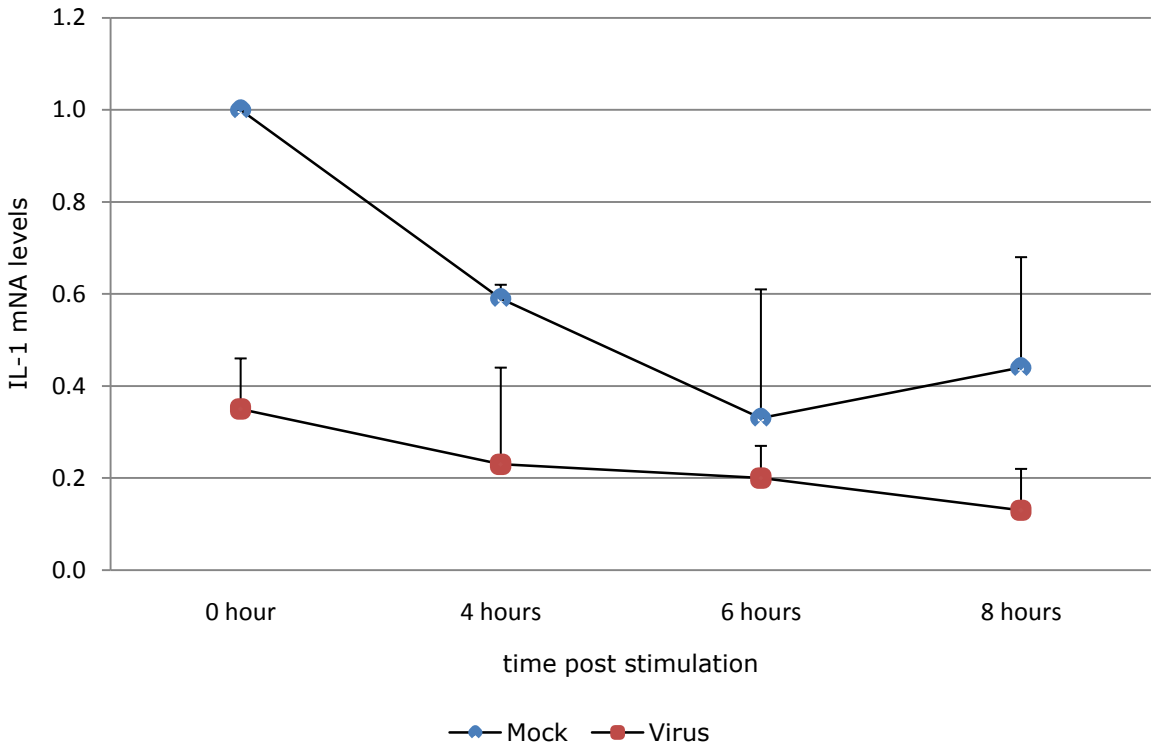

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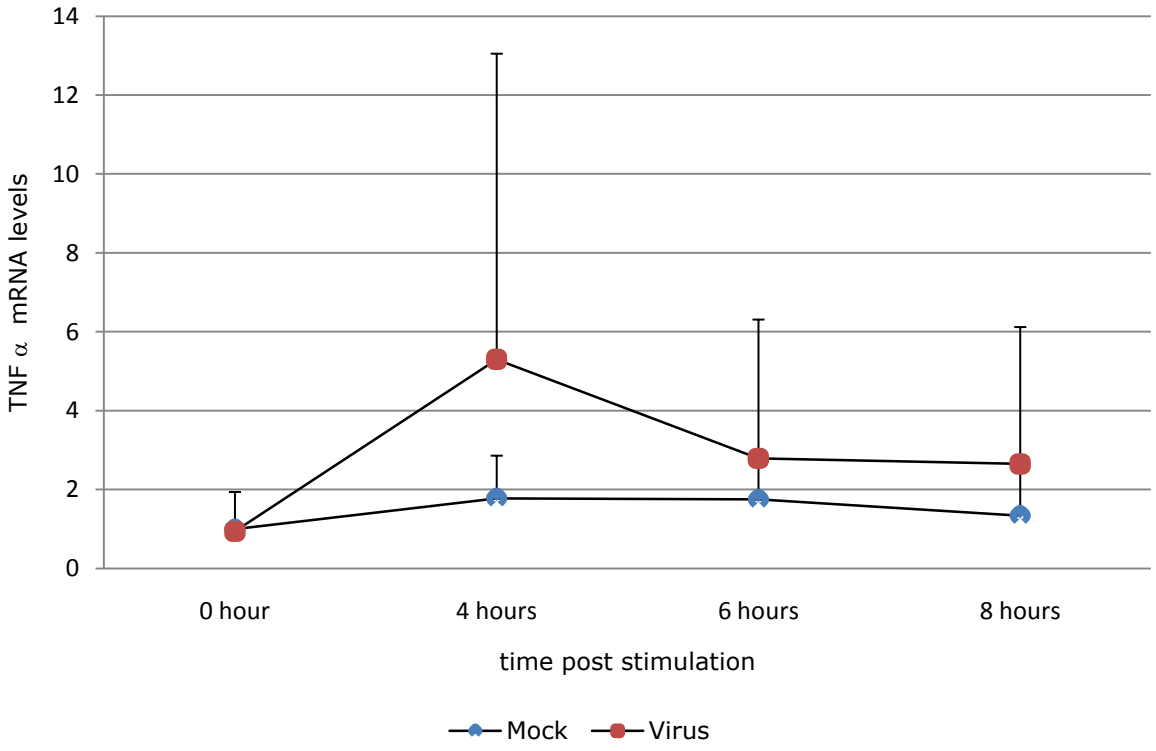

Figure 8 E

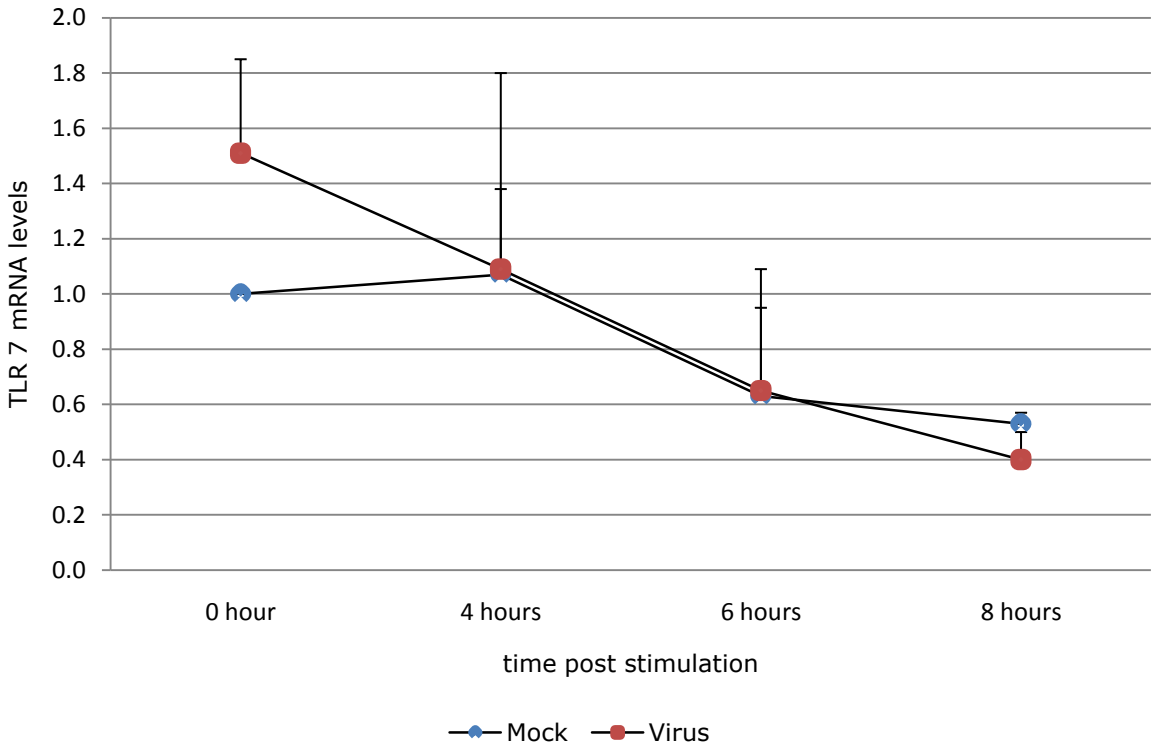
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
TLR 7 mRNA levels
0 hour
4 hours
6 hours
8 hours
time post stimulation
Mock
Virus

Figure 8 F

8. Acknowledgements

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And the uncle greets the master.

9. Curriculum vitae

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